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RESEARCH ARTICLE

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The method matters: The effect of handling time on cortisol level and blood parameters in wild cats

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Abstract

Blood analysis has recently become a popular tool to assess the welfare of the wild cats. However, the estimates of blood parameters may depend on the sampling method. We have tested (1) if the sampling procedure influences blood parameters and (2) what parameters are the most efficient in assessing the physiological status in wild cat species. We assessed the effect of handling time on red blood cells (RBC) and white blood cells (WBC) counts, the ratio of neutrophils to lymphocytes (N/L ratio), and serum cortisol level within 1 hr after the capture of the animal in six far-east wild cats (*Prionailurus bengalensis euptilura*). Also, we analyzed literature data in 17 cat species to assess the effect of place of study, type of immobilization, and handling time on WBC count and N/L ratio. Serum cortisol level varied significantly with the handling time. RBC and WBC counts were strongly affected by the handling time. N/L ratio was very robust and did not depend on the handling time. However, the analysis of literature data has shown that the prolonged handling time (over 1 hr) and the type of immobilization significantly influence the N/L ratio, whereas the WBC count does not depend on any of considered factors. We conclude that while most blood parameters of cats are affected by routine handling time, the N/L ratio does not vary if the samples are collected within 1 hr after the capture of the animal. All other tested parameters should be treated with caution.

KEYWORDS

cortisol, erythrocytes, handling time, leukocytes, ratio of neutrophils to lymphocytes, wild cats

1 | INTRODUCTION

Most of the wild cat species are rare and endangered (Nowell & Jackson, 1996), and there is a great need for efficient and reliable approaches to assess and monitor the physiological status of individuals (hormonal level, blood hematology and chemistry, immune competence) to predict the population dynamic. During the last 30 years, the use of blood profiles to estimate the welfare of wild cats in both captive and wild settings has become a usual practice among zoo veterinaries and field zoologists. At present, for 17 of the 38 wild cat species blood profiles have been obtained and described in scientific literature (Akuzawa, Mochizuki, & Yasuda, 1987; Brown, Lappin, Brown, Munkhtsog, & Swanson, 2005; Caro et al., 1987; Dunbar, Nol, & Linda, 1997; Erasmus, 2008; Kocan, Blouin, & Glenn, 1985; Marco, Martinez, Pastor, & Lavin, 2000; Prihirunkit, Salakij, Apibal, & Narkkong, 2007; Salakij, Salakij, Narkkong, Sirinarumitr, & Pattanarangsan, 2008a,b, b, 2009, 2010, 2011 and others). However, blood profiles can be influenced significantly by the sampling procedure, including capture method, type of anesthesia, and time to blood collection (Moen, Rasmussen, Burdett, & Pelican, 2010; Serieys et al., 2013).

Capturing wild cats is a labor-consuming procedure, not standardized, and may include trapping, chasing, capturing with the nets, darting with anesthetic drugs and other methods depending on cat species and study place (in the wild or captivity) (Beltrán, Delibes, Recio, & Aza, 1991; Caro et al., 1987; Dunbar et al., 1997; Naidenko, Pavlova, & Kirilyuk, 2014, 2018; Salakij, Salakij, Prihirunkit, Narkkong, & Pitakkingthong, 2010). The common procedure of collecting blood samples (handling time) in wild cats can be extremely stressful and implies the immobilization of animals with capture and anesthesia up to an hour both in captivity and in the wild (Akuzawa et al., 1987; Brown et al., 2005; Marco et al., 2000; Moen et al., 2010). Nevertheless, it is known that the basal glucocorticoid level must be sampled within 3 min of capture in birds and reptiles, after that, it increases dramatically (Loshchagina, Tsvey, & Naidenko, 2018; Romero & Reed, 2005). Blood profile is affected by stress too (Dhabhar, Miller, McEwen, & Spencer, 1995, 1996), so use of the total WBC number is

problematic. The values of this parameter can increase or decrease depending on animal species (see review in Davis & Maney, 2018; Davis, Maney, & Maerz, 2008). In particular, an elevated level of neutrophils combined with the low level of lymphocytes in blood observed in some cat species (Kocan et al., 1985; Serieys et al., 2013; Weaver & Johnson, 1995), has been related to the release of glucocorticoids (Mayer & Harvey, 2007; Thrall, Weiser, Allison, & Campbell, 2012). The quantification of blood parameters such as the ratio of neutrophils to lymphocytes (but not the total WBC number) may be used as a measure of immune cell reaction to stress in all vertebrates (Davis et al., 2008). However, it is difficult to distinguish the influence of the sampling procedure on blood profiles from the effects of tested factors such as species identity, reproductive status, season, diseases, social or environmental stress, and many others.

An important question is whether leukocyte profiles in wild cats vary depending on the handling time and the type of sampling procedure. To assess and monitor the health and welfare status of wild cats in the wild, we have to sort out the blood parameters that are less affected by the type of the procedure of collecting samples. One of such robust to stress parameters is the ratio of neutrophils to lymphocytes which can be estimated on blood smears and did not change significantly for a long time (more than 1 hr) after the stressful sampling procedure in some mammals and birds (Davis, 2005; Davis & Maney, 2018; Davis et al., 2008). However, the effects of sampling procedure and handling time on the neutrophils to lymphocytes ratio as well as other hematological parameters have not been studied in any wild cats.

In this study, we estimated different blood parameters and cortisol level in samples obtained during an hour after the beginning of animal physical capture in the Far-east wild cat (*Prionailurus bengalensis euptilura*). We assumed that: (1) the procedure of collecting blood samples changes adrenal activity in cats increasing cortisol level in blood serum; (2) the sampling procedure affects blood profile changing total number of blood cells (red blood cells [RBCs], white blood cells [WBCs]) in cats; (3) the N/L ratio is the most robust blood parameter to stress during the handling time in cats. In addition, we analyze available literature data on 14 cat species together with our own data on three cat species (domestic cat [*Felis catus*], bobcat [*Lynx rufus*], and far-east wild cat) to evaluate whether place of study (in the wild or captivity), type of immobilization (with or without anesthesia), and handling time (within an hour or more) influence the WBC number and the N/L ratio. We expected that (1) the N/L ratio would be more stable than the WBC count; (2) the handling time would affect these parameters stronger than the type of immobilization and place of study.

2 | MATERIALS AND METHODS

2.1 | Husbandry conditions and animals

The study was conducted in December of 2012–2013 using Joint Usage Center "Live collection of wild species of mammals" at A.N. Severtsov Institute of Ecology and Evolution (Tchernogolovka, Russia) situated 50 km northeast from Moscow, Russia (56°00′ N, 38°22′ E). In total, 35 adult animals of three cat species were used in experiments: domestic cat ($n = 16$; 7 ♂♂ and 9 ♀♀); far-east wild cat ($n = 14$; 5 ♂♂ and 9 ♀♀); bobcat ($n = 5$; 2 ♂♂ and 3 ♀♀). Cats were housed outdoor, under natural temperature and photoperiod conditions. Animals were kept separately in the wire-meshed cage with a wooden box as a shelter. The daily food ration consisted of chicken meat (0.5–1 kg per individual). Animals were fed six times a week and had water ad libitum. All animals were in apparent good health. More details of husbandry conditions were described earlier (Alekseeva, Antonevich, Erofeeva, & Naidenko, 2014; Glukhov & Naidenko, 2013; Naidenko & Erofeeva, 2004; Pavlova & Naidenko, 2008, 2012; Pavlova et al., 2014).

2.1.1 | Effect of handling time on cortisol level and blood parameters in far-east wild cats

We used six adult individuals of the Far-east wild cat (3 ♂♂ and 3 ♀♀) to estimate the effect of handling time on blood profiles and adrenal activity. We standardized the procedure of collecting blood samples (handling time) for all individuals. The handling time consisted of following steps: (1) entrance in a cage—beginning of an animal capture (with special jacket); (2) obtaining of the first blood sample under physical restriction of the cat within 3 min (1st point) after the beginning of the animal capture (this point we considered as basal level of adrenal activity because the blood samples was collected within 3 min in all tested animals); (3) injection of anesthesia (1 ml per animal of mixture 3:1 v/v of 2% solution of xylazine hydrochloride [Xyla, Interchemie, Castenray, Holland] and 10% solution of ketamine hydrochloride [Ketamin, Pharmanovo Gmbh, Hannover, Germany]) immediately after the first blood sampling; (4) obtaining of three blood samples at 20 min (2nd point), 40 min (3rd point), and 60 min (4th point) after the beginning of the animal capture. Overall, four blood samples were collected during an hour from every animal. The test was performed in morning (10:00–12:00 a.m.) during nonreproductive period in December of 2012 (Pavlova & Naidenko, 2012). The tests were completed within 2 days with three animals tested per day. The blood samples were collected from femoral vein. After that, blood samples were placed into two tubes for hormonal (Eppendorf tube; SSI, Lodi, California, USA) and hematological (about 0.25 ml into tube with EDTA; Aquisel S.L., Barcelona, Spain) analysis. In every blood sample, we analyzed three hematological parameters (RBC, WBC, the ratio of neutrophils to lymphocytes [N/L ratio]) and measured cortisol concentration. All data are shown in Table 1.

2.2 | Enzyme immunoassay

Blood samples for hormone analysis were centrifuged (20 min at the rate 6,000 rpm) immediately after collection and serum was transferred into new clear Eppendorf tubes, frozen and stored at −18°C until analysis. Serum samples were thawed and assayed for cortisol with EIA system. EIA was conducted using flatbed spectrophotometer Multiscan EX (ThermoElectron Corporation, Finland). The detection of cortisol level in serum samples was conducted using commercial kits for cortisol ("Immunotech," Moscow, Russia). The cross-reactivity of the cortisol antibody was 6% for prednisolone and < 1% for all other steroids tested. Serum samples were assayed in duplicates and concentrations were expressed as ng/ml of blood serum. The minimum level of

TABLE 1 The Effect of Handling Time on Changes of Serum Cortisol Level and Blood Parameters During an Hour after the Beginning of Animal Capture in far-East Wild Cats (Results of Repeated Measures ANOVA with Planned Contrasts, Mean ± SD, Range in Parentheses; $n = 6$ for all Points with Cortisol Concentrations; $n = 6$ for 3, 20, 40 min Points and $n = 5$ for 60 min Point with Blood Cell Numbers and Statistics)

	Time after the Beginning of Animal Physical Capture				
Parameters	3 min (Basal Level)	20 min (2 Point)	40 min (3 Point)	60 min (4 Point)	Statistics
Serum cortisol concentration (ng/ml)	616.5 ± 563.8	1006.7 ± 782.8	712.9 ± 740.8	811.9 ± 828.7	$\chi^2 = 8.8, P = 0.03$
	(73.7–1,375.0)	(149.8–2,122.0)	(74.5–1,944.0)	(43.8–1,900.5)	
Red blood cells, 10^{12}/l	9.4 ± 0.5	8.4 ± 0.6	8.0 ± 0.7	9.4 ± 1.7	$F = 29.3, P = 0.003$
	(9.0–10.1)	(7.4–9.1)	(7.2–9.2)	(8.2–12.3)	
White blood cells (10^9/l)	20.5 ± 4.4	12.1 ± 4.2	16.4 ± 10.8	21.5 ± 6.3	$F = 23.7, P = 0.005$
	(17.5–28.6)	(5.8–18.1)	(8.6–36.8)	(16.0–31.6)	
The ratio of neutrophils to lymphocytes (the N/L ratio)	2.6 ± 1.4	2.5 ± 1.2	2.8 ± 1.9	3.2 ± 2.0	$F = 0.4, P = 0.6$
	(1.2–5.0)	(1.2–4.4)	(0.5–6.4)	(1.5–6.6)	

detection was 1.81 ng/ml. Intraassay coefficient of variation for biological samples was 1.59 ± 0.65 % ($n = 24$) and interassay coefficient of variation was 1.62 ± 0.25 % ($n = 3$).

2.3 | Hematological analysis

Blood smears and blood cells counts were made within 20 min after blood collection. Red and white blood cells counting was conducted using automated veterinary hematological analyzer Abacus junior vet 1.22 (Diatron Messtechnik Gmbh, Budapest, Hungary). Fresh blood smears were fixed in 100% methanol immediately after drying up and stained with Romanovsky stain according to standard method (Bazhibina et al., 2005; Kozinets et al., 1998) shortly before the analysis. On a smear, a 100-cell differential leukocyte count was done manually using microscope Leica 5000D with software (Leica Microsystems, Switzerland) in the counting area (magnification × 1,000). Percentage of each leukocytes types was expressed in %. Total number of white blood cells of different type (lymphocytes, neutrophils, etc.) was calculated using the formula: absolute WBC number (counted by hematological analyzer) × percentage of WBC types/100.

2.3.1 | Effect of place of study, type of immobilization, and handling time on the number of white blood cells and the ratio of neutrophils to lymphocytes in wild cats

We used hematological literature data for 17 cat species (three species from this study and 14 species from papers; Supp. Table S1). For this experiment, we collected blood samples in captivity from adult individuals of domestic cat ($n = 16$), far-east wild cat ($n = 14$), and bobcat ($n = 5$) in December of 2013. Sampling method differed between three cat species. Far-east wild cats and domestic cats were physically restrained for blood collection. Bobcats were immobilized with the mixture of ketamine hydrochloride (Bremer Pharma GMBH, Bremerhaven, Germany) (6 mg/kg)/xylazine hydrochloride (Rometar; Bioveta, Ivanovice na Hané, Czech Republic) (0.36 mg/kg). Further blood sampling and hematological analysis were performed as described above for the far-east wild cat.

We categorized the procedure of collecting blood samples from all cat species according to three parameters: (1) place of study (in the wild or in captivity), (2) type of animal immobilization (anesthesia or without anesthesia), and (3) handling time (within 1 hr or more than 1 hr). We pooled all types of anesthesia due to small sample sizes, but in the most cases it was ketamine/xylazine hydrochloride combination (Akuzawa et al., 1987; Beltrán et al., 1991; Brown et al., 2005; Kocan et al., 1985; Marco et al., 2000; Salakij et al., 2009).

For all tested cat species, we considered the total WBC number and the N/L ratio that was calculated based on mean values of neutrophils and lymphocytes counted on the slide. All data are shown in Table 2. For papers where the data were presented for females and males separately, we calculated the weighted averages. The standard error of mean was recalculated to the standard deviation using the formula: $SD = SE \times \sqrt{N}$. If there were no standard deviation or standard error of mean in a paper, we estimated the standard deviation based on the range as SD = (maximum – minimum)/4 (Zar, 2013).

2.4 | Statistical analysis

The log-transformed level of the blood cortisol confirmed to the normal distribution (Shapiro-Wilk's W test, $P > 0.05$), and we analyzed the cortisol dynamics using repeated-measures analysis of variance (ANOVA) at four levels of the "time" factor: 3 (baseline point), 20, 40, and 60 min after the beginning of manipulations with the animal (test points) followed by the Tukey post-hoc test. For cortisol level comparison, we used η^2 as an effect size statistics to measure the strength of an effect (Cohen, 1988; value of 0.01 indicates a small effect, 0.06 indicates a medium-sized effect, and 0.14 indicates a large effect).

The numbers of blood cells did not meet assumptions of normality (Shapiro-Wilk's W test, $P < 0.05$) and homoscedasticity and could not be transformed. Therefore, we applied rank transformation of the data. We compared the ranked blood cells numbers at test points of the handling time and method of immobilization with the basal level using planned contrasts, followed by Dunnet post-hoc test with the basal level point as a control point. In the cell number comparisons,

TABLE 2 The Effect Place of Study, Type of Immobilization, and Handling Time on the Number of White Blood Cells (WBC) and the Ratio of Neutrophils to Lymphocytes (N/L) in Wild Cats

Species	Type of Immobilization	Handling Time	Place of Study	Number of Tested Animals	WBC, 10^{*9}/l	N/L	Authors
Bobcat (*Lynx rufus*)	Anesthesia	More than 1 hr	In the wild	11	10,60	3,82	Kocan et al. (1985)
Bobcat (*Lynx rufus*)	Anesthesia	More than 1 hr	In the wild	11	13,90	7,73	Serieys et al. (2013)
Bobcat (*Lynx rufus*)	Anesthesia	Within 1 hr	In captivity	10	7,30	2,67	Serieys et al. (2013)
Bobcat (*Lynx rufus*)	Anesthesia	Within 1 hr	In captivity	5	13,87	3,03	Our data
Canadian lynx (*Lynx canadensis*)	Anesthesia	More than 1 hr	In the wild	20	9,30	6,46	Moen et al. (2010)
Canadian lynx (*Lynx canadensis*)	Anesthesia	Within 1 hr	In captivity	13	10,00	3,74	Moen et al. (2010)
Canadian lynx (*Lynx canadensis*)	Anesthesia	Within 1 hr	In captivity	22	9,10	4,87	Weaver and Johnson (1995)
Cheetah (*Acinonyx jubatus*)	Anesthesia	Within 1 hr	In the wild	17	12,50	3,10	Caro et al. (1987)
Clouded leopard (*Neofelis nebulosa*)	Anesthesia	Within 1 hr	In captivity	17	14,30	4,33	Salakij et al. (2008b)
Domestic cat (*Felis catus*)	Without anesthesia	Within 1 hr	In captivity	–	12,50	1,76	Meyer and Harvey (2007)
Domestic cat (*Felis catus*)	Without anesthesia	Within 1 hr	In captivity	16	18,05	1,39	our data
European wild cat (*Felis silvestris*)	Anesthesia	Within 1 hr	In captivity	20	14,67	2,10	Marco et al. (2000)
Far-east wild cat (*Prionailurus bengalensis euptilura*)	Without anesthesia	Within 1 hr	In captivity	14	16.09	2,00	our data
Fishing cat (*Felis viverrina*)	Anesthesia	Within 1 hr	In captivity	13	7,07	2,92	Prihirunkit et al. (2007)
Flat-headed cat (*Prionailurus planiceps*)	Anesthesia	Within 1 hr	In the wild	2	13,90	1,68	Salakij et al. (2008a)
Florida panther (*Puma concolor*)	Anesthesia	Within 1 hr	In the wild	48	12,19	2,23	Dunbar et al. (1997)
Iberian lynx (*Lynx pardina*)	Anesthesia	More than 1 hr	In the wild	16	17,20	7,19	Beltrán et al. (1991)
Iriomote (*Prionailurus iriomotensis*)	Anesthesia	Within 1 hr	In the wild	17	12,72	6,33	Akuzawa et al. (1987)
Jungle cat (*Felis chaus*)	Without anesthesia	Within 1 hr	In captivity	8	12,58	2,94	Salakij, Prihirunkit, Salakij, Narkkong, and Tongthainun (2011)
Leopard (*Panthera pardus*)	Anesthesia	Within 1 hr	In captivity	17	13,02	3,60	Salakij et al. (2009)
Leopard cat (*Prionailurus bengalensis*)	Without anesthesia	Within 1 hr	In captivity	12	9,50	0,67	Salakij et al. (2010)
Lion (*Panthera leo*)	Anesthesia	Within 1 hr	In captivity	72	12,71	4,18	Erasmus (2008)
Pallas cat (*Otocolobus manul*)	Anesthesia	Within 1 hr	In the wild	15	11,10	2,74	Brown et al. (2005)
Pallas cat (*Otocolobus manul*)	Anesthesia	Within 1 hr	In captivity	9	9,40	2,02	Brown et al. (2005)

we excluded one blood sample of one male at the fourth point (60 min) from all analyses and calculations because of blood clotting in this sample. At the same time, the cortisol estimation for this sample was valid and included in analyses.

We analyzed the WBC number and the N/L ratio from literature data using General linear models with place of study, type of animal immobilization, and handling time as factors in the model.

All statistical analyses were performed with STATISTICA, v. 13.0 (StatSoft, Tulsa, OK). All tests were two-tailed with a significance level of 0.05.

2.5 | Animal welfare

All experimental procedures did not have any visible adverse effects on the animals. After the collecting blood samples the individuals moved and behaved normally. No drop in the body mass was recorded in subsequent weighings. No special permission for use of cats in behavioral researches is required in Russian Federation. The relevant committee that regulates research regarding animals in A.N. Severtsov Institute of Ecology and Evolution of the Russian Academy of Sciences The Commission on Regulatory of Experimental Research (Bioethics Commission) of IEE RAS was created only in 2017. The study was conducted in accordance with the ASAB/ABS (2012), Guidelines for the Treatment of Animals in Behavioural Research and Teaching and with the laws of Russian Federation, the country where the research was conducted.

3 | RESULTS

3.1 | Effect of handling time on cortisol level and blood parameters in far-east wild cats

The serum cortisol concentration varied significantly within an hour after the beginning of capturing procedure in far-east wild cats (repeated-measures ANOVA with log-transformed data, $F = 4.3$, $P = 0.02$, $N = 6$; $\eta^2 = 0.46$ corresponds to large effect): the level of cortisol increased in the first 20 min in all tested individuals (Tukey post-hoc test, $P = 0.049$; Figure 1) (Table 1). Then cortisol concentration decreased and became similar to the 3 min (basal) level at 40 and 60 min points (Tukey post-hoc test, $P > 0.1$).

The RBC number was strongly affected by the handling time (Table 1): as compared with the basal level, it decreased significantly by the 20 min point (Dunnet post-hoc test, $P = 0.01$) and became even lower at the 40 min point ($P = 0.0004$).

The WBC number also varied with the handling time: it decreased by the 20 min point in all individuals (Dunnet post-hoc test, $P = 0.037$), then it increased and at 40 min-point became similar to the basal level ($P = 0.16$).

In contrast to RBC and WBC numbers, the N/L ratio was not affected by handling time (Table 1).

3.2 Effect of place of study, type of immobilization, and handling time on the number of white blood cells and the ratio of neutrophils to lymphocytes in wild cats

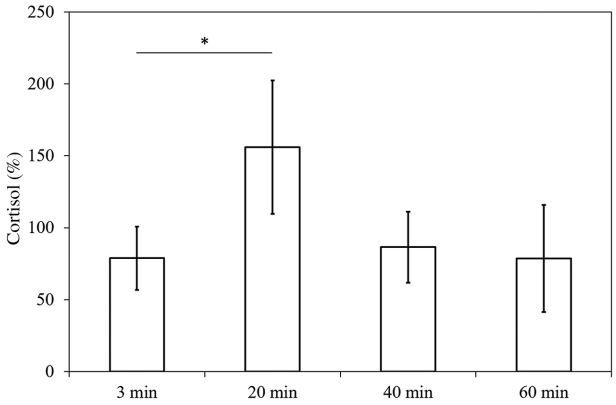

FIGURE 1 The effect of handling time on serum cortisol level during an hour after the beginning of animal capture in far-east wild cats (see statistics in the text; * corresponds to $P < 0.05$). Because of high individual variability, the cortisol level in the graph is calculated for each individual as a percentage of its average value during the experiment

Based on literature data, we analyzed 25 hematological profiles for 17 cat species, including the domestic cat (Table 2). The total WBC number did not depend significantly on handling time ($F_{1,20} = 0.2$, $P = 0.7$) or place of study ($F_{1,20} = 0.8$, $P = 0.4$); it was slightly and insignificantly lower in studies with using of anesthetic drugs ($F_{1,20} = 2.9$, $P = 0.1$).

The type of immobilization as well as handling time affected the N/L ratio significantly. The N/L ratio was greater in studies with prolonged handling time (within 1 hr vs. more than 1 hr; $F_{1,20} = 19.0$, $P = 0.0003$; Figure 2A) and with anesthesia ($F_{1,20} = 6.5$, $P = 0.02$; Figure 2B). The study place (in the wild or in captivity) did not affect the N/L ratio ($F_{1,20} = 0.04$, $P = 0.8$; Figure 2C). The maximum range of the N/L ratio were obtained in cat species in which blood samples were obtained using anesthesia over 1 hr (all these studies were conducted in the wild and animals were captured by traps; Table 2). The lowest value of the N/L ratio was in studies when blood samples were obtained by physical restrain, without anesthesia, within 1 hr (all these studies were conducted in captivity).

4 | DISCUSSION

This is the first study where dynamic of blood parameters (RBC and WBC numbers, N/L ratio) and adrenal activity (serum cortisol concentration) were monitored together during an hour after the beginning of animal physical capture. It is also the first analysis of literature data performed to evaluate effect of handling time, type of immobilization, and place of study on the WBC number and the N/L ratio in wild cats.

4.1 | Effect of handling time on cortisol level and blood parameters in far-east wild cats

According to our first prediction, the cortisol level increased almost twice by the 20th minute after the beginning of capture procedure in all individuals of the far-east wild cat. In this species, full activation of adrenal system by injection of adrenocorticotropic hormone

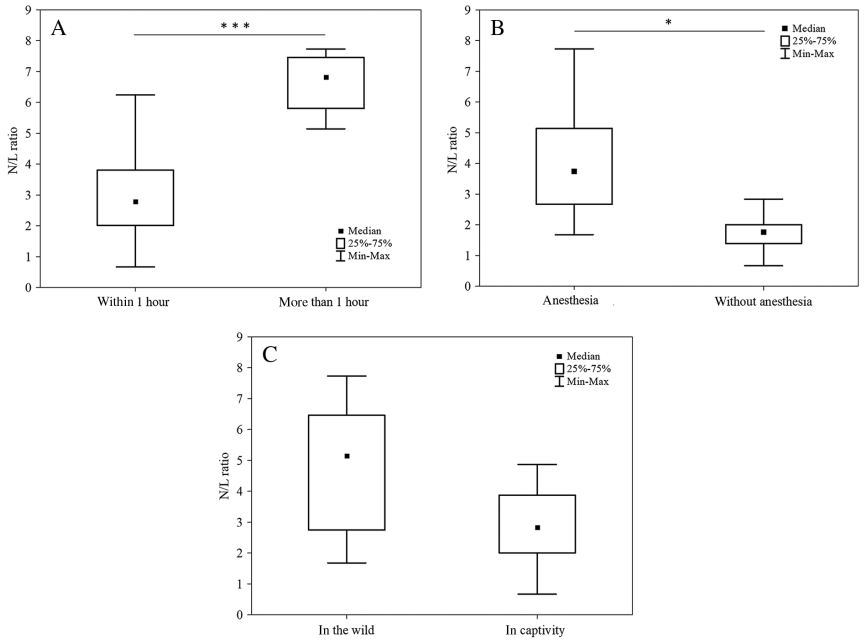

FIGURE 2 The effect of handling time (A), type of immobilization (B), and place of study (C) on the ratio of neutrophils to lymphocytes (N/L ratio) in wild cats (GLM, * corresponds to $P < 0.05$; *** corresponds to $P < 0.001$)

(ACTH) elevates cortisol level three and half times in an hour after ACTH procedures (Pavlova & Naidenko, 2008). This result is consistent with studies of rats (Muir & Pfister, 1987) and water skinks (*Eulamprus heatwolei*) (Langkilde & Shine, 2006) showed that stressful events such as transportation, handling, increase corticosterone level in blood (in 3–15 min) comparing with basal level. Thus, our results prove that standard method of blood collection in small cats (physical restrain with immediate injection of anesthesia and collecting blood samples) is stressful for far-east wild cats.

Next, the total WBC number decreased fast and dramatically. This result is consistent with studies of birds, mice and rats showed that prolonged (over 30 min to 3 hr period) stressful events (such as transportation, handling, ACTH injection) induced a significant decrease in WBC number (Davis, 2005; Dhabhar et al., 1995, 1996; Scope, Filip, Gabler, & Resch, 2002). We found that number of WBC declined even faster, after 20 min, but then returned to the basal level. Lymphopenia and neutrophilia is usually caused by release of glucocorticoids and occurred in animals as a response to various forms of stress (Mayer & Harvey, 2007). However, in our case, we did not observe such changes in blood parameters in far-east wild cats within an hour. Probably, they would develop later. The decline of WBC number at 20 min could be induced by the shock from capture procedure as well as WBC shift from the circulating to the marginal pool (Mayer & Harvey, 2007).

Basal level of RBC in far-east wild cats was higher than in most cat species (Caro et al., 1987; Kocan et al., 1985; Prihirunkit et al., 2007; Salakij, Prihirunkit, Narkkong, Apibal, & Tongthainun, 2008b), but very similar to leopard cat (*P. bengalensis*), which was physically immobilized also without anesthesia within an hour (Salakij et al., 2010). We showed that RBC number decreased in 20 min after the beginning of capture procedure. Five minutes of 4% isoflurane anesthesia resulted in a slight decrease in the erythrocyte parameters in rats and ferrets (Thrall et al., 2012). Nevertheless, it is unclear why RBC number became lower after the first sampling.

Thus, the procedure of blood sampling affects blood profile: it changes total numbers of blood cells (RBC, WBC) by the 20th minute after the beginning of animal capture that is consistent with our second prediction. These facts should be taken into consideration when the parameters are used for the estimation of physiological status in wild cats, especially if the information about species normal value is unavailable.

The N/L ratio fluctuated insignificantly within 2.5–3.2 during an hour in far-east wild cats, although it increased slightly over this period. The value of N/L ratio was slightly higher than in domestic cats (about 1.8, Mayer & Harvey, 2007). However, our results are consistent with the data for various mammals: generally, neutrophils are twice as numerous as lymphocytes (Thrall et al., 2012). The lack of significant effects of handling time on the N/L ratio agrees with our third prediction and suggests that the N/L ratio measured within an hour after the beginning of stressful manipulation is not affected by routine handling, capture and anesthesia in far-east wild cats. Early works in some mammals and birds demonstrate similar stability of this blood parameter during 2 hr after stress procedures (Burguez, Ousey, Cash, & Rossdale, 1983; Davis, 2005; Davis et al., 2008).

4.2 | Effect of place of study, type of immobilization and handling time on the number of white blood cells and the ratio of neutrophils to lymphocytes in wild cats

According to literature data, the mean WBC count and the N/L ratio vary strongly among cat species (7.1–18.1 $\times$ 10^9/l and 0.7–7.7, respectively; Table 2). At the same time, the available data for the total number of leukocytes are more or less within the norms for domestic cats (5.5–19.5 $\times$ 10^9/l) (Mayer & Harvey, 2007). However, the standards for

domestic cats should be used with caution for other species because these values may vary strongly even among close species. The effects of different factors on the WBC count in animals only add to the confusion, since there is no information about the normal range of leukocyte numbers in most of species (Davis et al., 2008; Johnstone, Reina, & Lill, 2012). Zoo database includes data on many cat species but no information how these data were obtained (anesthesia type, handling status, individual health, and reproductive status). As mentioned above, the change in the WBC number can occur both in a high and in a low way and proceeds very fast (Dhabar et al., 1995, 1996; Scope et al., 2002; Davis, 2005), which complicates the interpretation of the data.

High basal N/L ratio (more than 3) may be an evidence of inflammation, stress, or lack of welfare. Low level of neutrophils (lower than 1.0) can indicate the presence of virus infections like feline leukemia virus and feline immunodeficiency virus or some bacterial infections and other diseases (Bazhibina et al., 2005; Mayer & Harvey, 2007; Thrall et al., 2012).

The analysis of literature data showed that the variation in N/L ratio could be determined by the factors of handling time and immobilization method. The maximum level of N/L ratio was observed for cat species in which blood samples were collected using anesthesia over 1 hr. All studies with long handling time were conducted in the wild and traps were used for the capture of animals. In this case, we have typical combination of increased neutrophil and decreased lymphocyte numbers in blood. It is a well-known phenomenon determining stress leukogram (Mayer & Harvey, 2007), probably induced by capture stress. Mean N/L ratio did not exceed 3.2 when the sampling was conducted within 1 hr in all cat species, independently of method of immobilization and place of study (Table 2). Literature data support our results for far-east wild cats concerning stability of the N/L ratio within about 1 hr. Thus, based on analysis of literature data we also conclude that the N/L ratio does not vary if the samples are collected within 1 hr after the capture of the animal, so this valuable index may be used for estimation of long-term stress (Davis & Maney, 2018) and physiological status (Thrall et al., 2012) in cats as it is used in other mammals and birds. At the same time, all other tested parameters should be used with caution. The stability (based on literature data) of leukocytes number in cats over the different handling time (more and less than 1 hr) and anesthesia type may be neglected by our experimental data in far-east wild cats: this parameter changed within 20 min after the beginning of experimental procedures.

5 | CONCLUSIONS

The standard method of the procedure of collecting blood samples in small cats (physical restrain with immediate injection of anesthesia and collecting blood samples) is stressful for far-east wild cats and leads to elevation of their serum cortisol levels. Moreover, blood sampling procedure affects the total number of blood cells (RBC, WBC) within 20 min after the beginning of animal capture. Thus, these blood parameters should be used in wild cats with caution. Our results as well as literature data confirm that the most stable blood parameter over the short-term (within an hour) stress procedures is the N/L ratio.

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